Reference: *Biol. Bull.*, **147**: 369–385. (October, 1974)

ANATOMY OF THE INNERVATION AND NEUROMUSCULAR JUNCTIONS OF THE RADULAR PROTRACTOR MUSCLE OF THE WHELK, *BUSYCON CANALICULATUM* (L.)

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J. C. Herrick (1906, on page 731) suggested that the radular protractors of the channeled whelk "would make fine material for physiological experiments upon molluscan muscle because of their length and regularity." We are in the midst of such experiments using the radular protractor muscles of *Busycon canaliculatum* (Hill, 1958; Hill, 1962; Hill, 1970; Hill, Marantz, Beattle and Lockhart, 1968; Hill, Greenberg, Irisawa and Nomura, 1970; Sanger, 1973; Sanger and Hill, 1972, 1973a). The innervation of the buccal muscles of *Busycon canaliculatum* was described in some detail by Herrick (1906) whose objective was to explain the *modus operandi* of the buccal apparatus. His paper is a model of clarity, giving the results of four to five weeks of dissecting and experimenting in the summer of 1905. We could not improve on his comments, nor upon his description of the macroscopic anatomy of the musculature, but his description of the innervation was incidental to his study of the functional anatomy of the buccal muscles. The time has now come for a more detailed description of the *innervation* not only of the radular protractor muscle but also of the whole buccal apparatus.

In this report we also present the results of our observations on the fine structure of the nerves and of the neuromuscular junctions in the radular protractor muscles. A preliminary report of this work was presented last summer at the General Meetings of the Marine Biological Laboratory (Sanger and Hill, 1973b).

Materials and Methods

Whelks, *Busycon* (*Busycotypus*) *canaliculatum* (Linné 1758), were obtained from the supply department, Marine Biological Laboratory in Woods Hole, Massachusetts. The animals were dissected under a low powered binocular microscope, sometimes with the aid of methylene blue injection of blood vessels. The wall of the proboscis was cut through ventrally and pinned back. Since both muscles and nerves were pigmented, the innervation could be blocked out by careful dissection without staining. The smaller blood vessels were not apparent except after injection through the main vessel of methylene blue solution (0.5% methylene blue in distilled H_2O diluted in sea water to the desired color contrast; usally 1 : 5).

Whelks were fixed and processed for electron microscopy as previously described (Sanger and Hill, 1972). Thick sections for the light microscope, 1 to 3 microns, were cut with an LKB Ultratome III and stained with paraphenylenediamine (Estable-Puig, Bauer and Blumberg, 1965).

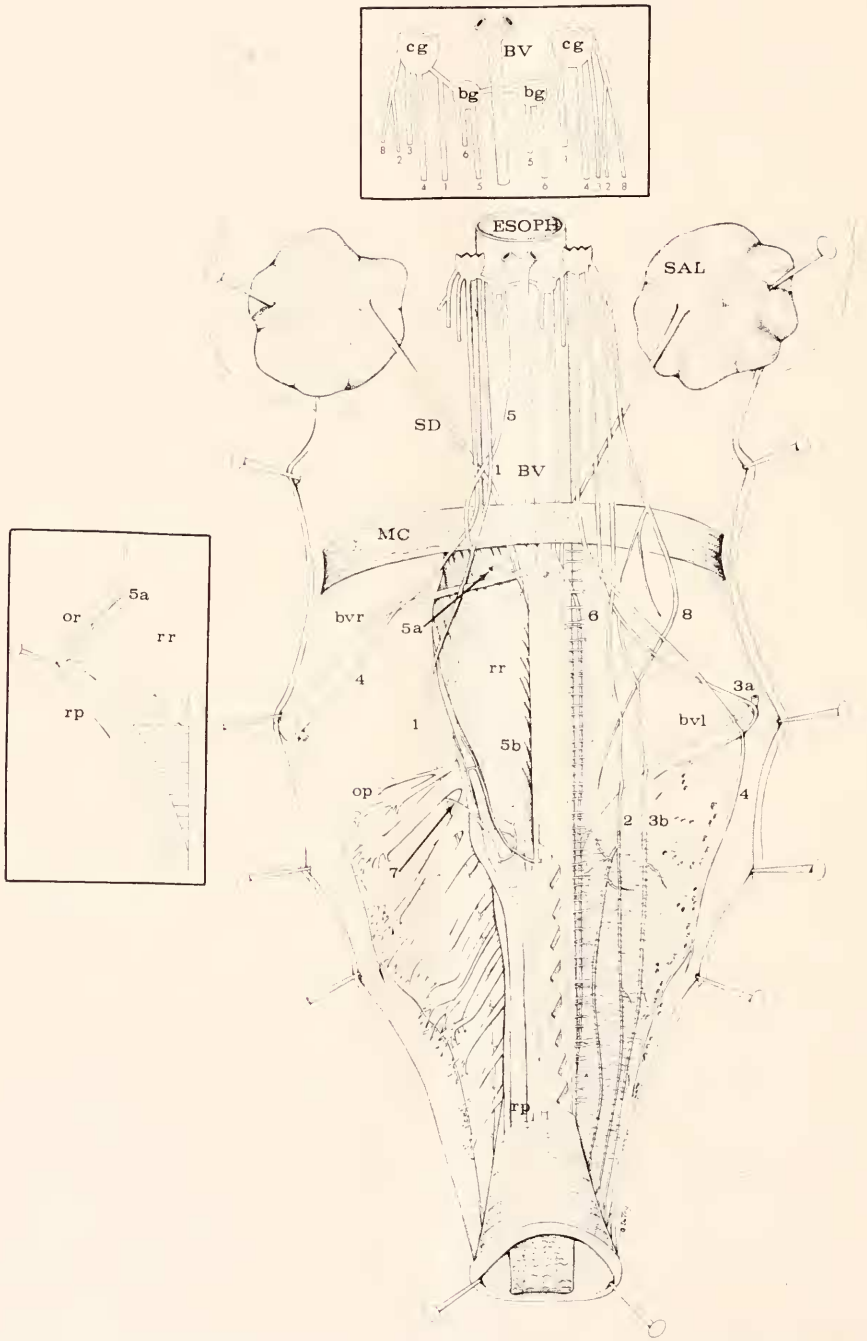

FIGURE 1. Innervation of the proboscis musculature of *Busycon canaliculatum;* 1–8 = Proboscis nerves. See text for details; BV, chief proboscis artery; ESOPH, esophagus; MC,

Results

We have adhered rigidly to Herrick's terminology (1906) except in the rare instances in which we found that we must disagree with his identification as noted below. [We will *not* reiterate Herrick's description of the macroscopic anatomy of the muscles.]

A semi-diagrammatic representation of the innervation of the buccal apparatus and proboscis wall in *Busycon canaliculatum* based on a dozen or more dissections was prepared and sketched (Fig. 1). We have found individual variations in the finer branches of the nerves, but not in the main trunks. The drawing represents a usual distribution of the finer branches.

The proboscis nerves arise from the buccal ganglia (bg), from the cerebral ganglia (cg) and from the cerebro-buccal connective. In the upper insert of Figure 1 the nerves are numbered for clarity, using Herrick's (1906) *distal* numbers except in the case of nerve 8 (which he did not describe).

Innervation of the proboscis wall is from the cerebral ganglia by nerves 2, 3, 4 and 8. Nerves 2 and 8 arise together from the cerebral ganglia. Nerve 8 soon appears as a branch which first runs ventrally to the muscular cup (MC) and then innervates the white muscle fibers of the proximal dorsal proboscis wall. The other branch, nerve 2, first runs anteriorly (along with all the proboscis nerves) to the muscular cup (MC). All nerves except 6 then pass ventral to the muscular cup (MC). [Most nerves in fact continue more or less on the ventral aspect of the proboscis, but they appear lateral in the drawing, which is a representation of a preparation in which the proboscis wall has been slit ventrally and then pinned out flat, exposing the buccal apparatus]. Nerve 2 then runs anteriorly, ventral to the large median root of the radular retractor (rr). It then runs dorsal to the odontophore protractors (op) along the dorsal proboscis wall, but it is not attached to that wall until it reaches the region of the antrior red fiber layer of the proboscis wall. It then passes under the red fiber layer and gives off branches to the proboscis wall as far as the region of the mouth. Nerve 3 runs along laterally to 2, but somewhat more centrally (ventrally) and provides a branch (3a) which meets and then runs parallel to blood vessel "bvl" or "brr" (for clarity, each nerve is diagrammed only on one side, of the proboscis, but all are symmetrical). All fine branches from 3 supply the proboscis wall. Nerve 4 is the most central (ventral) of the proboscis wall nerves. It is difficult to avoid cutting this nerve when making a ventral incision into the proboscis. To summarize: nerves 2, 3 and 4 arise from the cerebral ganglia; all supply the probscis wall; and all run down to end in the region of the mouth. Since the innervation is symmetrical, and since there is a dorsoventral sequence 2–3–4: the cylindrical tube of the proboscis wall is evidently divided into six longitudinal fields of innervation (probably overlapping).

Innervation of the esophagus is supplied symmetrically from the buccal ganglia by nerve 6 on each side. Nerve 6 runs anteriorly with the other proboscis nerves from a buccal ganglion (bg) to the muscular cup (MC). It then pene-

muscular cup; SAL, salivary gland; SD, salivary duct; bg, buccal ganglion, bvr, right branch from BV; bvl, left branch from BV; cg, cerebral ganglion; op, odontophoral protractor; or, odontophoral retractor; rp, radular protractor; rr, radular retractor.

trates the muscular cup along with the salivary gland duct (SD). After emerging from the muscular cup, nerve 6 runs forward along the esophagus, usually passing under the white muscle fibers that tie the esophagus to the dorsal proboscis wall. On the anterior (red) region of the esophagus, nerve 6 runs with the salivary gland duct (SD) and blood vessel (bvl) providing many branches to the red anterior esophagus.

Nerves 1, 5 and 7 supply the muscles of the buccal apparatus. Since nerve 7 is a branch of nerve 1, this means that the mechanism controlling the movements of the radula is worked by both an outflow from the buccal ganglion (nerve 5) and an outflow from the cerebro-buccal connective (nerve 1) on each side. Nerve 1 arises from the cerebro-buccal connective, usually close to the buccal ganglion, runs anteriorly ventral to the muscular cup (MC), passes ventral to the large median root of the radular retractor (rr), and then runs dorsal to each odontophoral protractor (op). Nerve 7 arises as a large branch of nerve 1, generally just anterior to the most proximal odontophoral protractor. It then branches to innervate the proximal ends of the radular protractors and to supply the transverse muscular sheet which binds the anterior portion of the buccal apparatus. Additionally, a stout branch turns back into the radular retractor (rr). Thus nerves arising with trunk 1 supply odontophoral protractors (op), radular protractors (rp), and a portion (at least) of the radular retractors. Herrick (1906) also reported that nerve 1 supplied the odontophoral pretractors and that nerve 7 (a branch of nerve 1) supplied the radular protractors and the transverse muscle sheet. In his drawing, nerve 7 may also be seen to return in the direction of the radular retractors, although he attributed the innervation of the radular retractors to nerve 5.

Nerve 5 arises from the buccal ganglion, runs with nerve 1 as far as the median root of the radular retractors and then passes within the median root. It soon gives off a branch (5a) which passes laterally in the midst of the median root, giving off small branches, and then enters the odontophoral retractor (or) (see left insert on the diagram). The main nerve (5b) spirals forward in the median root of the radular retractor, giving off small branches and continues anteriorly and dorsally in the radular retractor. Small branches from nerve 5b often seem to anastomose with small branches from the recurring branches of nerve 7, which reenter the radular retractor.

Electrophysiological experiments were carried out (by K. Kuwasawa) to determine if fibers of 5 and 7 actually crossed over. It was found that some motor fibers of 5 did cross over into 7 and vice-versa. The fact that there can be a pathway for excitation at the anastomoses (which are not uncommon throughout the buccal innervation) makes physiological investigation of the innervation more complex, since cutting a given nerve as it emerges from its ganglion would not necessarily isolate it from CNS outflow. Here for example, Herrick has attributed control of the radular retractor to nerve 5, yet stimulation of the innervation of antagonists of the radular retractors can also excite the retractors e.g., stimulation of nerve 7 to the radular protractors).

There is a large artery (BV) entering the proboscis, which gives off two symmetrical lateral branches (bvl and bvr). Injection of methylene blue revealed their continuity with the central arteries in the radular protractors (rp) (Fig. 2)

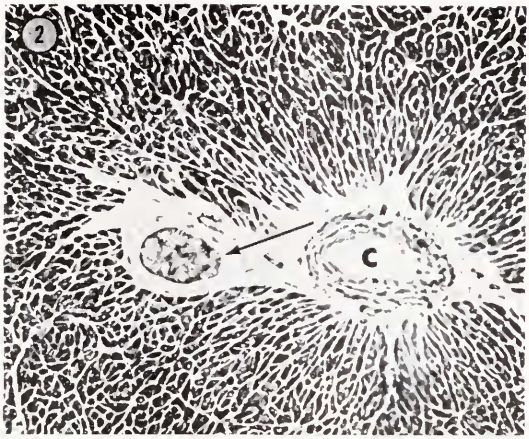

FIGURE 2. A light micrograph demonstrating the relationship of the central blood vessel (c) and main nerve trunk (arrow) in a cross section of the radular protractor muscle.

and in the odontophoral protractors (op). All three vessels remain large until they branch into the tissues around the mouth. All of the buccal muscles are richly supplied with small branches, and since the circulation is open, injection with methylene blue quickly stains the entire space around the muscles in the proboscis.

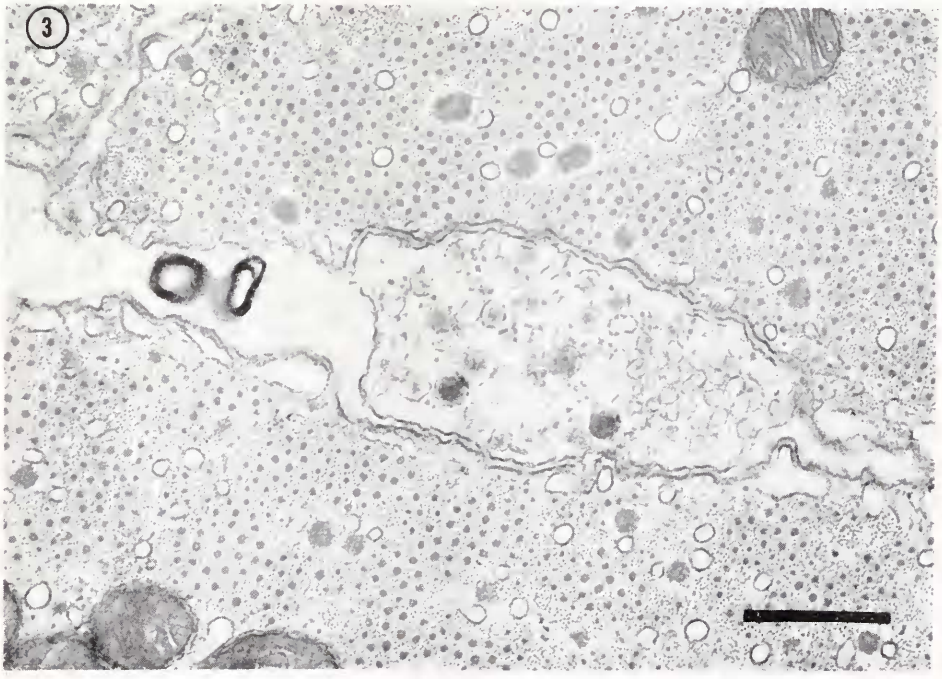

FIGURE 3. A nerve ending between two muscle cells; scale = 0.5 micron.

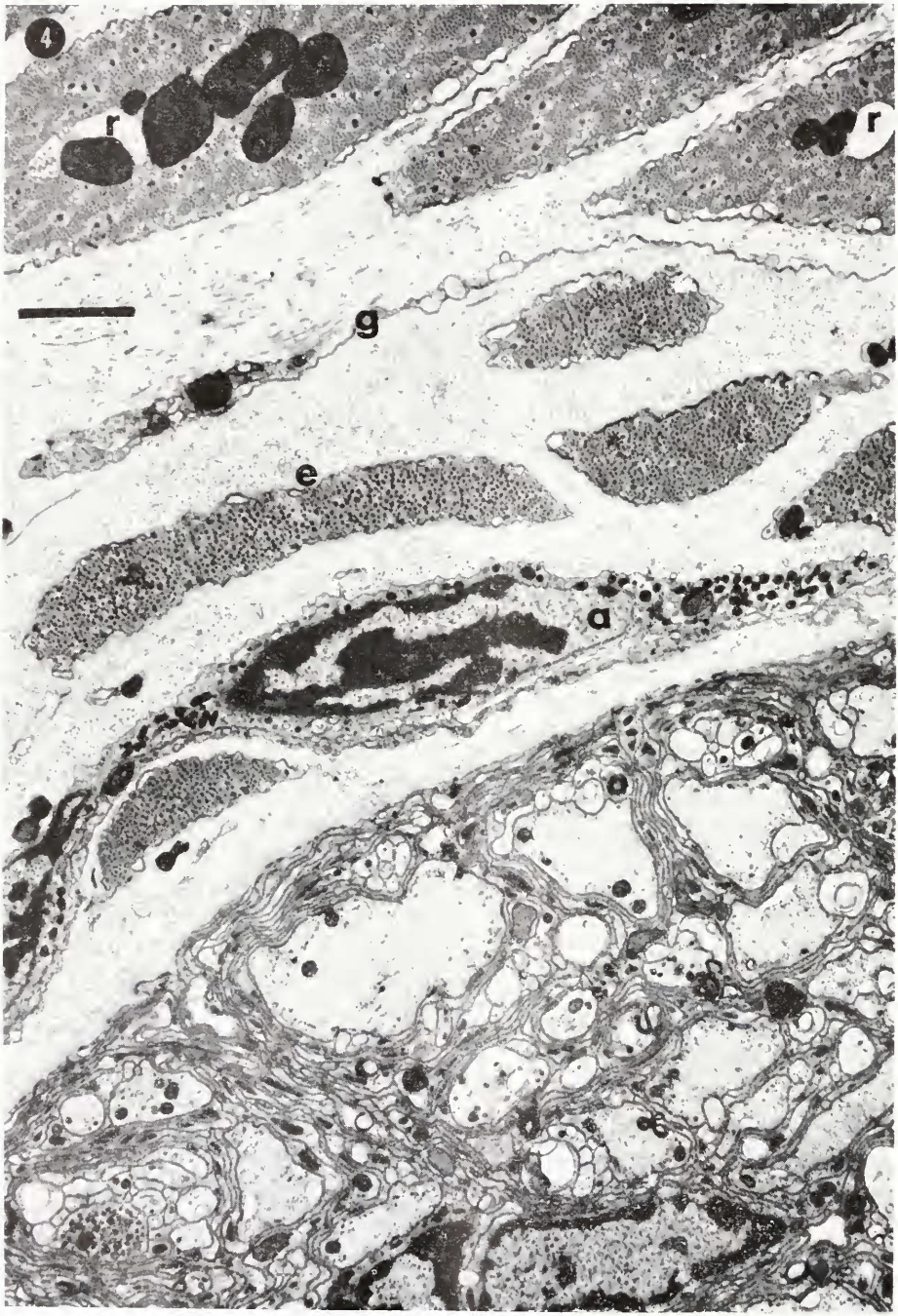

FIGURE 4. An electron micrograph of the nerve trunk surrounded by a discontinous coat of epineural muscle (e), amobocytes (a) and glial cells (g) embedded in collagen. The radular protractor muscle cells (r) are also indicated; scale = 2.0 microns.

Nerves and neuromuscular junctions in the radular protractor muscles: A branch of nerve 7, along with a branch of the blood vessel enter the proximal portion (with regard to the CNS) of the radular protractor muscle which is attached to the odontophore cartilage. The main trunk of the branch of nerve 7 runs parallel to the central artery in the mid part of the muscle (Figure 2). As the nerve proceeds anteriorly towards the attachment of the muscle to the radular sac, it subdivides to form smaller trunks. These smaller trunks continue to subdivide until finally single axons are seen among the muscle cells, where neuromuscular junctions are formed. Occasionally, a single nerve ending is observed forming a junction with two adjacent cells (Fig. 3) (Graziadei 1966).

The main branch of nerve 7 in the radular protractor muscle is surrounded by a discontinuous layer of amoebocytes, glial and epineural muscle cells (Figure 4). These cells are embedded in a thick collagen matrix. The collagen matrix appears to be divided into two unequal layers. The adjacent layer (about 2500 Å thick) is composed of collagenous fibers which for the most part wind circumferentially around the nerve trunk. The outermost layer (about 8 microns in width) is composed of mostly collagenous fibers which are arranged parallel to the long axis of the nerve trunk and epineural muscle cells. The cells embedded in the collagenous neural sheath do not form any continuous barrier to separate the axons from the extracellular space of the radula muscle. The main nerve trunk is about 40 microns in diameter and contains several hundred axons ranging in size from less than a micron to about 8 microns in cross section. The axons contain various arrays of microtubules and neurofilaments. In transverse section, the larger axons are rather irregular in outline and are generally found close to the surface of the trunk. The axons are associated with cells termed sheath cells (Rogers, 1968) or glial cells (Amoroso, Baxter, Chiquoine and Nisbet, 1964; Nicaise, Pavans de Ceccatty and Baleydier, 1968; McKenna and Rosenbluth, 1973). Hereafter in accordance with the nomenclature of Amoroso, Baxter, Chiquoine and Nisbet, 1964, these cells will be referred to as glial cells (Rogers, 1969). In the lamellar extensions of the glial cells are large membrane-bound inclusions which vary greatly in size, density and shape (Fig. 5). The inclusions ranged in diameter from 0.3 to 0.8 microns. These glial bodies are comparable to those described by Barrantes (1970) in the Agentinian slug (*Vaginula soleiformis*). The lamellar extension of the glial cell with its characteristic large membrane-bound vesicles is frequently located near neuromuscular endings. As also noted by Rogers (1969), processes of glial cells not associated with axons are often encountered between the muscle cells (Fig. 4).

Smaller subdivisions of the main nerve trunk are encountered away from the central portion of the muscle bundle (Fig. 5). These smaller trunks (less than 10 microns in diameter) are no longer surrounded by a coat of epineural muscle. The thickness of the collagenous coat and the number of axons in these smaller trunks are also reduced. The maximum diameter of the largest axon is also smaller; generally none larger than one micron. Figure 5 is an illustration of a small nerve branch about 5 microns in diameter which contains about seventy axons.

In large and small nerve bundles granular and agranular vesicles (similar to synaptic vesicles in neuromuscular endings) are observed in various axons (Figs. 5, 6). In these nerve bundles, an axon appears to contain only one type of vesicle. However, both granular and agranular synaptic vesicles are observed in the same

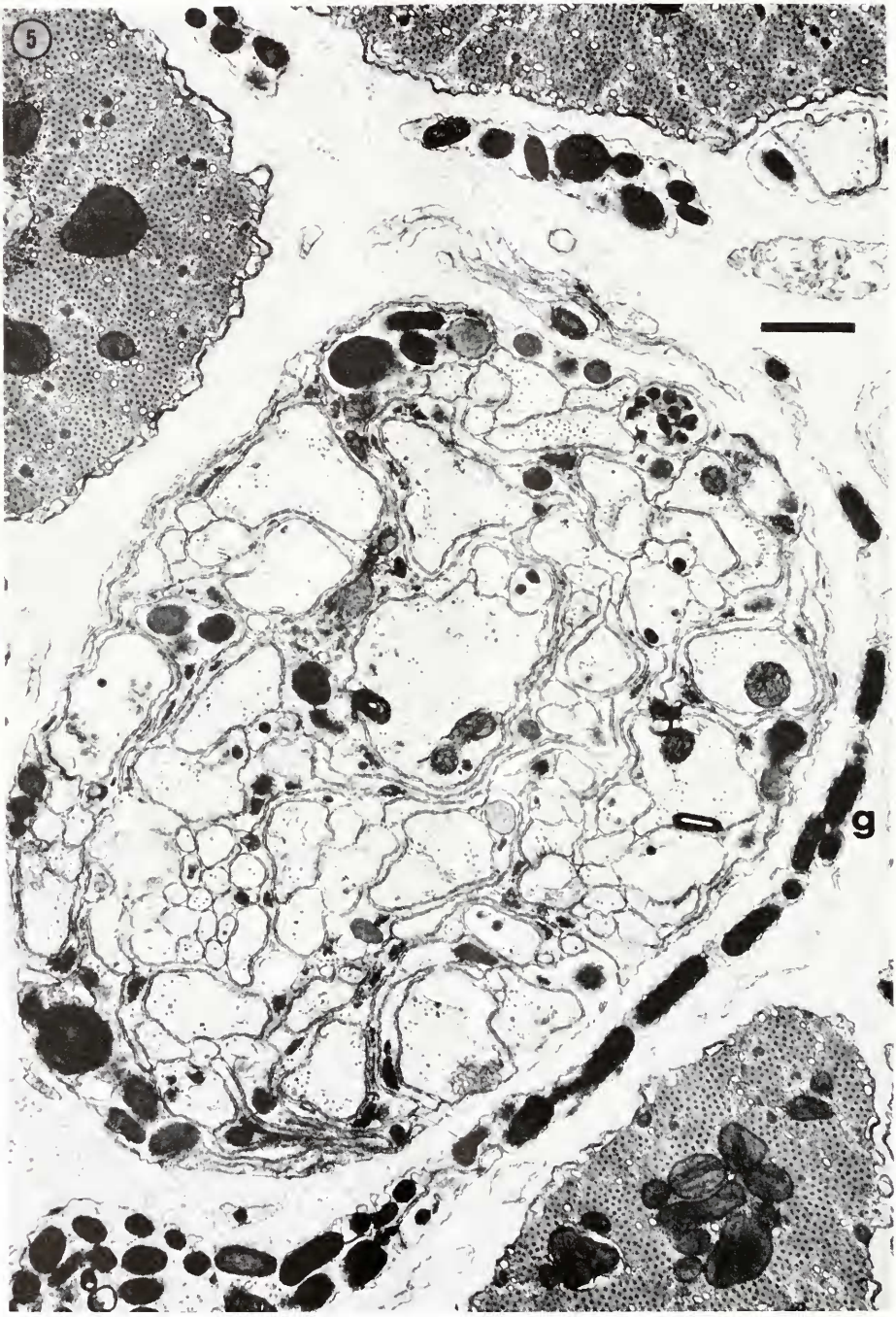

FIGURE 5. A cross section of a smaller nerve trunk among the radular protractor muscle cells. Note the absence of the epineural muscle cells and the meandering process of a glial cell (g); scale = 0.5 micron.

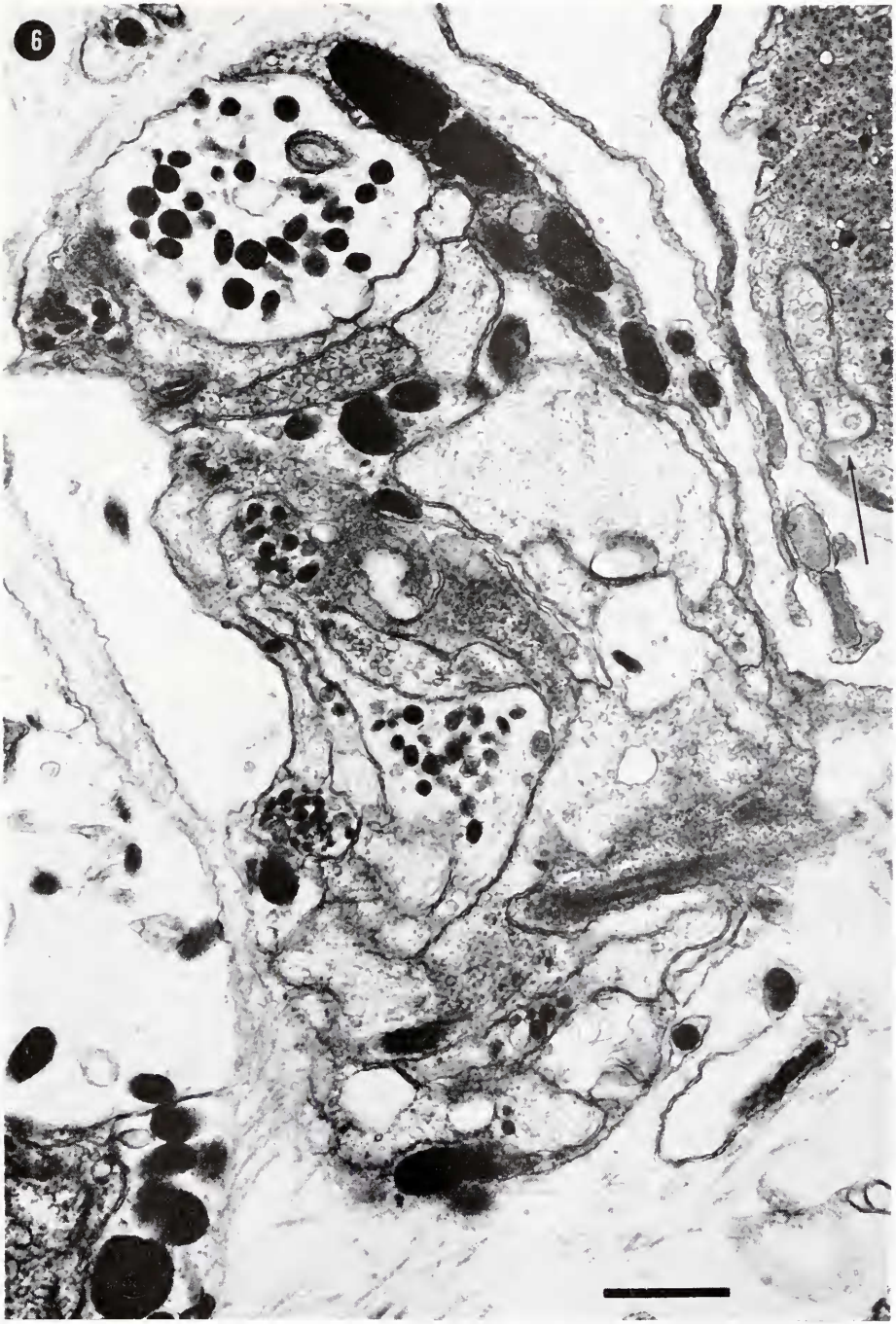

FIGURE 6. Many of the axons in this small nerve trunk contain uniform populations of synaptic vesicles. A nerve ending (arrow) is surrounded by a process of the muscle cell; scale = 1.0 micron.

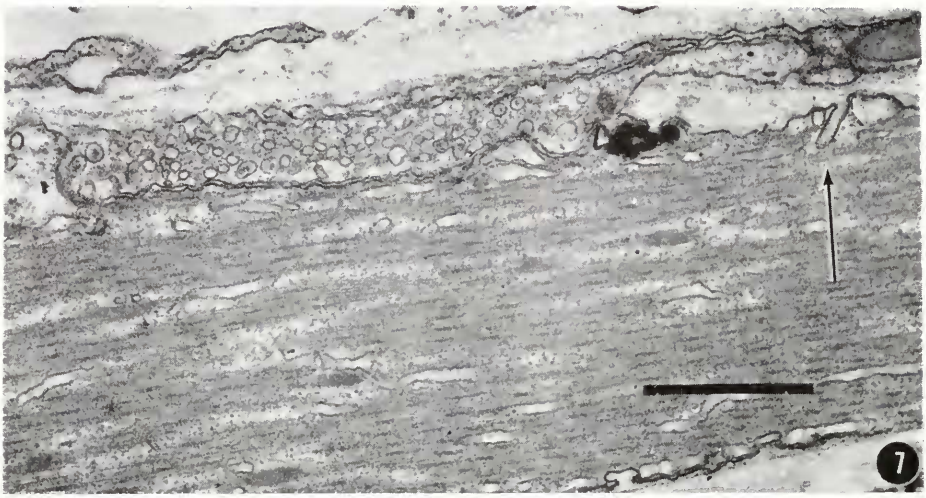

FIGURE 7. The initimate juxtaposition of the nerve ending and muscle cell is illustrated here. A sarcolemmic tubule is also clearly pictured (arrow); scale = 1.0 micron.

neuromuscular junction (Figs. 7, 8, 9). The muscle cell and nerve are separated by a gap of about 150–200 Å. The muscle is intimately associated with the nerve ending but there is no specialized membrane involution in the junctional area such

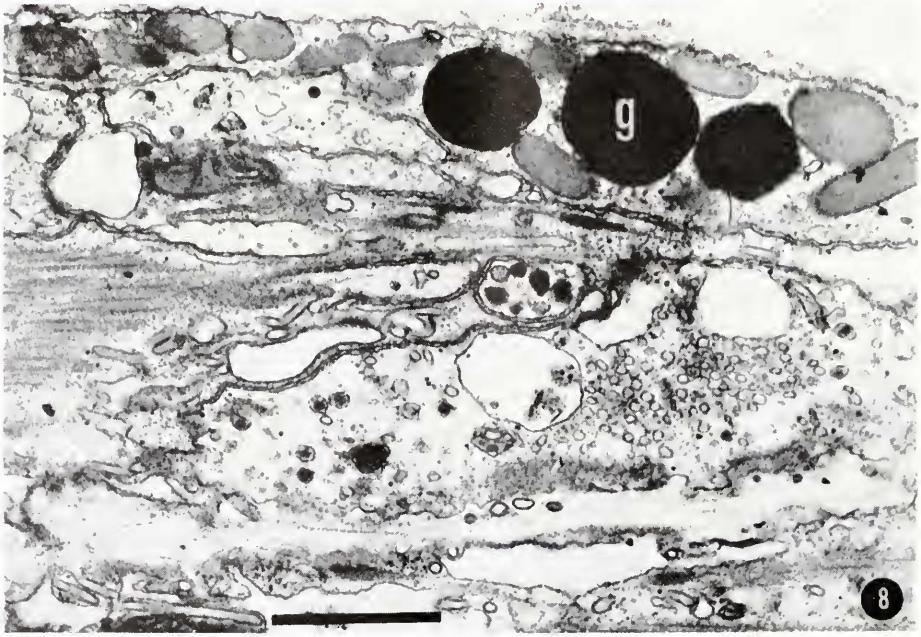

FIGURE 8. A nerve ending containing a mixture of dense and clear synaptic vesicles. The glial cell (g) process contains many large granules; scale = 1.0 micron.

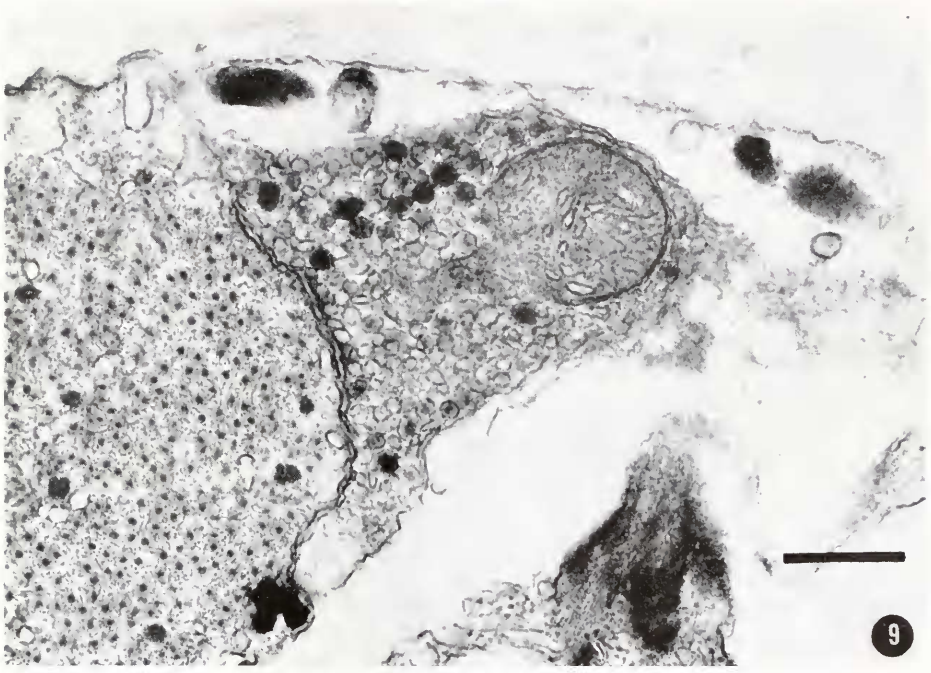

FIGURE 9. A cross section of a nerve ending with a mixture of dense and clear vesicles; scale = 0.5 micron.

as is observed in vertebrate fast twitch skeletal muscle. The association of nerve and muscle is comparable to that observed in vertebrate and molluscan smooth muscle (McKenna and Rosenbluth, 1973). Frequently, part of the nerve ending is ensheathed by a process of the muscle cell it is innervating (Figure 6).

There is a decided segregation in the size of the synaptic vesicles. The agranular vesicles ranged in size from 500 to 1000 Å with a mean diameter of 630 Å (based on the measurement of 400 vesicles). The granular vesicles ranged in size from 800 to 1350 Å with a mean diameter of 970 Å (based on a count of 200 vesicles). The granular vesicles varied in electron density. Some vesicles were completely filled with dense material while others were only partially filled. Nevertheless, both types of granular vesicles had about the same diameter, 800–1350 Å.

The two extremes of neuromuscular junctions are illustrated in Figures 10 and 11. Figure 10 indicates a nerve ending where less than ten per cent of the synaptic vesicles are granular while Figure 11 illustrates an ending where ninety per cent of the synaptic vesicles are granular. Nerve endings with intermediate proportions of granular and agranular vesicles are also observed.

DISCUSSION

A good deal of past attention has been devoted to the function of buccal muscles in the feeding cycle of prosobranch gastropods, and some heat has been generated by past controversies which perhaps did not take sufficient account of the extreme diversity to be found in the anatomy of these muscles in this group. A definitive

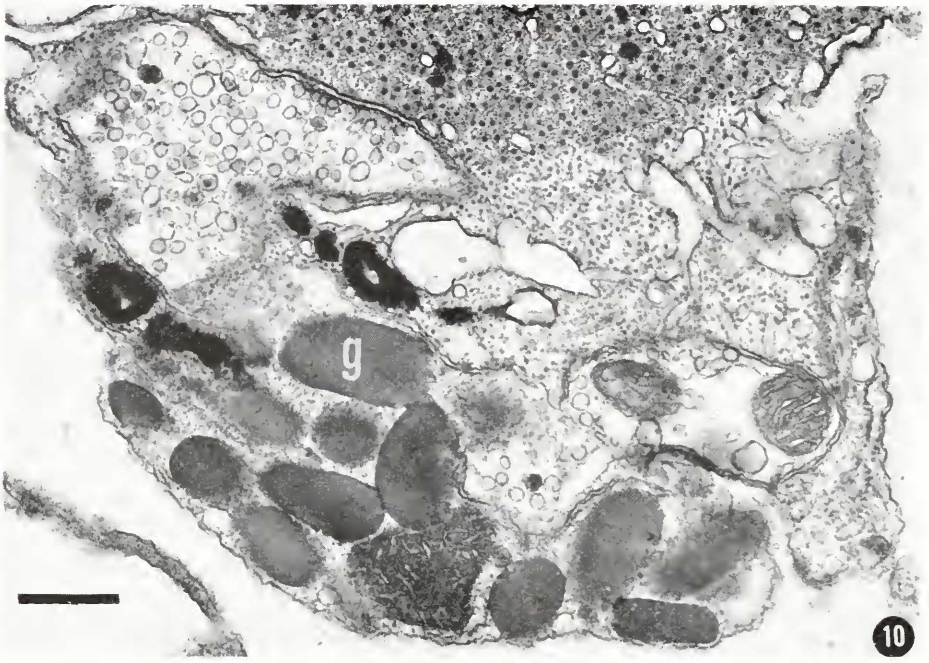

FIGURE 10. A nerve ending where many of the vesicles are clear. Note the intimate association of the glial cell (g) and nerve ending; scale = 0.5 micron.

account of the feeding cycle is available in Chapter 8 of *British Prosobranch Molluscs* (Fretter and Graham, 1962). There are apparent homologies among the muscles in a number of genera, but there are also profound modifications linked with modifications in use of the radula.

The *modus operandi* of the whole odontophoral mechanism of *Busycon* was described by Herrick (1906). As we re-examined the mechanism we felt the need for a redescription of the innervation, which was only one facet of Herrick's work. This was mainly because the responses of the muscles to stimulation of the nerves did not altogether correspond to those to be expected from Herrick's description. We now feel the discrepancies may principally be attributed to the anastomoses described above. Otherwise we have only improved in some details on Herrick's description, but we have provided an integrated picture of the whole buccal innervation.

Hoyle (1964) noted in a review some ten years ago the scarcity of work reported on the ultrastructure of molluscan nerve endings. In the ensuing decade much less than a score of papers have been devoted to this subject (see summary of references in Heyer, Kater and Karlsson, 1973). Even fewer of these studies have been done on systems which are also being investigatd by physiological and pharmacological experiments. The radular protractor muscle of *Busycon canaliculatum* is being investigated in three different ways: physiologically, pharmacologically and ultrastructurally (Hill, 1958; Hill, 1970; Hill, Greenberg, Irisawa and Nomura 1970; Sanger and Hill, 1972; Sanger and Hill, 1973a, 1973b;

Sanger, 1973). Our intention has been to combine the results from these three avenues of experimentation to study regulation of the contractile properties of the smooth muscle at several levels. The radular protractor muscle is of particular interest because it can be induced to show rhythmicity in the presence of acetylcholine and tryptamine (Hill, 1958) and perhaps could be used as a model of cardiac rhythmicity.

The two extreme types of nerve endings observed in this present study appear very similar to serotoninergic nerve endings (containing mostly granular vesicles) and to cholinergic (containing mostly clear vesicles) nerve endings. The nerve endings containing mostly clear vesicles are like those of cholinergic nerve endings in vertebrate smooth muscle (Richardson, 1964). Even there, occasional dense synaptic vesicles were observed by Richardson among the many clear vesicles. These clear or agranular synaptic vesicles might be acetylcholine storage sites while the dense or granular vesicle may be 5-hydroxytryptamine storage sites.

Welsh and Moorhead (1959), using a fluorescence assay method, identified the presence of 5-hydroxytryptamine in the radular muscles of *Busycon canaliculatum*. The concentration was rather low (0.09 μg/g) compared to that in the pooled ganglia (9.2 μg/g), and the origin may well have been from vesicles in the nervous tissue of the radular muscles. This is, of course, speculation since the radular protractor muscle has not yet been studied by subcellular fractionation or by the use of the Falck method. Acetylcholine has not been identified chemically in radular muscles or nerves, although a number of authors have found acetylcholine-equivalent by bioassay in gastropod cardiac muscle (summarized by Welsh, 1956).

The immediate interest of the observation that the nerve endings may have cholinergic and aminergic vesicles lies in the fact that a mixture of opposing synthetic neurohumors, acetylcholine (ACh) and serotonin (5-HT), induce strong maintained rhythmicity in the radular protractor (Hill *et al.*, 1970). Acetylcholine depolarizes and induces a contracture, which is not a "catch" since the muscle relaxes along with the repolarization which follows washing out of the acetylcholine. However, if the muscle is treated with tryptamine while still depolarized with acetylcholine, repolarization is gradual and accompanied by a regular oscillation which induces mechanical rhythmicity. The phenomenon is being investigated, with the objective of discovering the mechanism of the rhythmicity but it may have nothing to do with the natural rhythmic rasping function of the radula, since to all appearances the muscles of the buccal apparatus function in a phasic non-spontaneous motor unit organization (Herrick, 1906). However, it remains possible that acetylcholine may be the excitatory neurotransmitter. When the radular protractor is driven by stimulation of its nerve (Hill, 1962) 10^{-8} M ACh may increase amplitude of twitches by one-third. Michael J. Greenberg (Florida State University) and his students in the Experimental Invertebrate Zoology Course (MBL) recently found that ACh antagonist blocked twitches caused by stimuli to the nerve (M. J. Greenberg, personal communication). ACh antagonists also block ACh-induced contraction (Hill, 1970). However, tryptamine or 5-HT also increases amplitude of the twitches when the muscle is driven by stimulating its nerve, with a threshold around 10^{-8} M. Furthermore, high concentrations of 5HT (10^{-3} M) predispose the muscle to rhythmicity, in the sense that

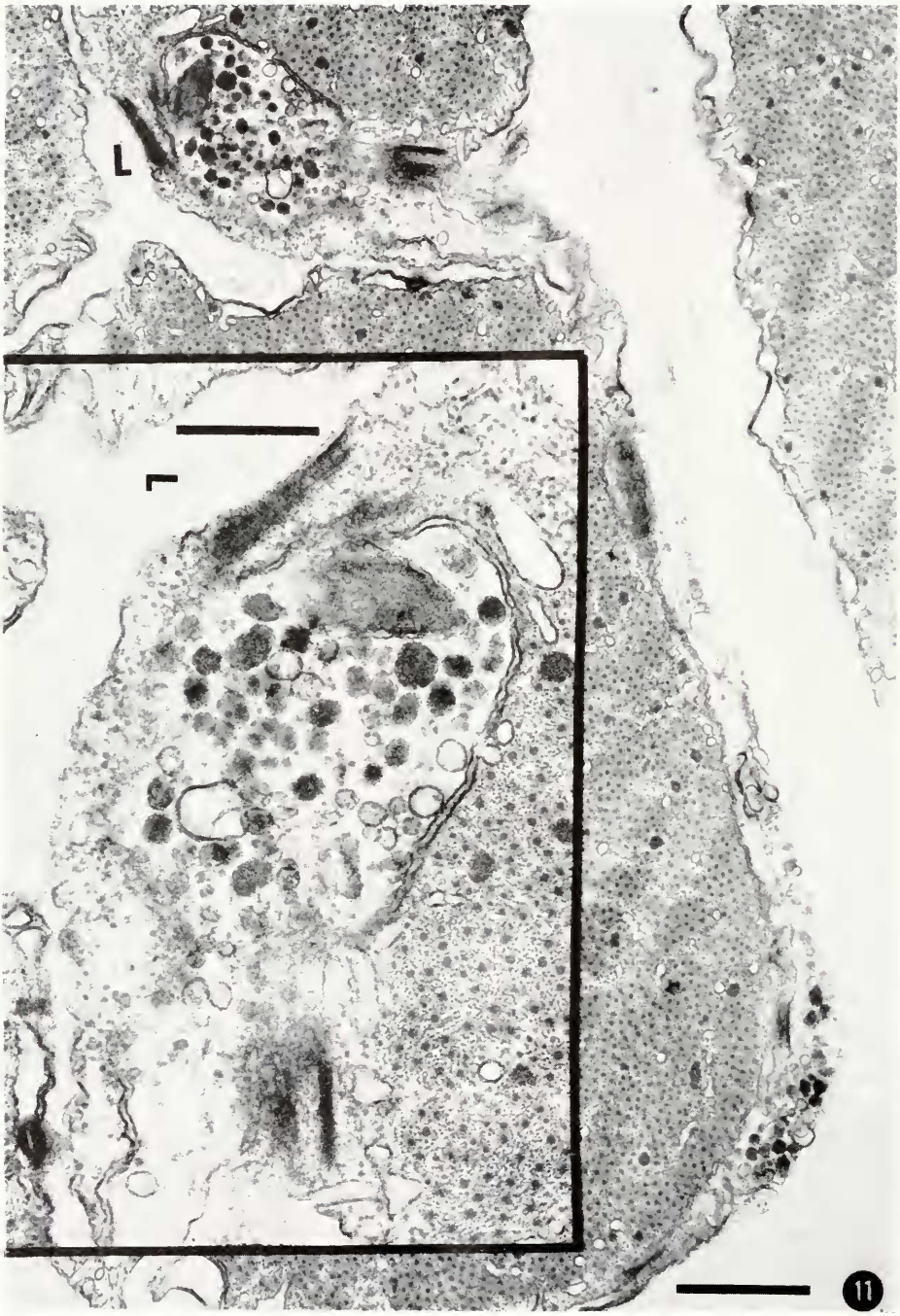

FIGURE 11. Two nerve endings where most of the vesicles contain dense material. The scale in the large picture equals 1.0 micron. The insert is a higher magnification (scale = 0.5

subsequent stimulation of the nerve or directly of the muscle evokes oscillatory responses, and subsequent stimulation with acetylcholine or KCl may evoke a contracture with superimposed rhythmicity. Thus if the vesicles do indeed contain ACh and 5HT it remains possible that there may be two types of excitatory synapses

The variation in types of synaptic vesicles in nerve endings reported here in the radular protractor muscle, a phasic muscle, is similar to that reported in a "catch" muscle, the anterior byssus retractor muscle (ABRM) (McKenna and Rosenbluth, 1973). Several workers have proposed the existence of two types of nerve endings in that catch muscle. One ending is cholinergic and that nerve ending produces depolarization and contraction, by the release of acetylcholine, which leads to the "catch" state (*i.e.*, the persistent contraction after the muscle has repolarized). A second type of ending is serotoninergic (containing dense vesicles) and by the release of serotonin immediately relaxes the muscle from the "catch" state (Twarog, 1967). In contrast, the radular protractor muscle does not demonstrate any "catch" phenomena—yet it has a similar display of nerve endings as in the "catch" muscle (McKenna and Rosenbluth, 1973). These observations demonstrate a similar ultrastructure of the neuromuscular endings of two dissimilar types of muscles, a "catch" muscle and a phasic muscle.

As indicated by McKenna and Rosenbluth (1973) the mixing of both granular and agranular synaptic vesicles in nerve endings is observed rather frequently in invertebrates. The presence of both types of synaptic vesicles in the same nerve endings has been reported in several other molluscan species (Barrantes, 1970; Dougan and McClean, 1970; Heyer, Kater and Karlsson, 1973). This mixing of different types of vesicles in the same nerve ending raises further questions about their identification and function. Can nerve endings have more than one neurotransmitter? Are the vesicles in the small axons identical to those in the nerve endings? Are some of the clear vesicles in the nerve endings just depleted dense vesicles? Further work-involving serial sections of the nerve endings and small nerve trunks as well as the isolation and identification of the various granules—is certainly needed to elucidate these questions.

We wish to particularly acknowledge collaboration by Mrs. Else Froberg and Dr. Kiyoaki Kuwasawa. The diagram of the innervation is based on dissections by R. B. Hill, D. Spring, L. Vargish, F. Froberg and K. Kuwasawa. The drawing by G. DeVry is based on sketches by E. Froberg, K. Kuwasawa and M. Parmenter.

The investigation was supported by research grant NS 08352 from the National Institute of Neurological Diseases and Stroke and by the National Science Foundation (GB1001 and GB5598) (R.B.H.). Research for this publication was also supported by NIH Grant No HL 15835 to the Pennsylvania Muscle Institute and the National Science Foundation (IG-73-3) (J.W.S.). Please send reprint requests to J.W.S.

micron) of the upper nerve ending. The change in orientation in the insert is indicated by the letter "L."

Summary

1. A detailed and integrated picture of the whole buccal innervation of *Busycon canaliculatum* is presented.

2. The results of our observations on the fine structure of the nerves and of the neuromuscular junctions in the radular protractor are reported and discussed. Each radular protractor muscle is innervated by a nerve arising from the cerebrobuccal connective. The nerve trunk enters the proximal end of the muscle and runs parallel to the long axis of the muscle bundle. A layer of connective tissue and epineural muscle cells surrounds the trunk. Subdivisions of the main nerve trunk branch laterally into the muscle bundle losing their epineural muscle coat. Further subdivisions of the branches produce single axons which can be observed among the muscle cells, but no specialized motor nerve endings were observed. Within the nerve endings are two types of synaptic vesicles: agranular (clear) and granular (dense). The granular vesicles are larger, ranging in diameter from 800 to 1350 Å (mean 970 Å). The clear vesicles vary in diameter from 500 to 1000 Å (mean 630 Å). The ratio of agranular to granular vesicles within a single nerve ending varies widely. When one type of vesicle predominates in an ending, then that ending comes to have a resembance to a cholinergic or to a serotoninergic nerve ending.

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